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A QUICK MICROTEST TO DETERMINE SENSITIVITY OF GERMS TO ANTIBIOTICS

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(Clinical Weekly)
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Gerd Poetschke
Ortwin Hanke

Walch (1951) described a "quick bacteriological test to determine the resistance to antibiotics" in this same journal. The test is based on the microculture technique described by Poetschke and Bommer (1950).

Simultaneously with Walch and independently of him a similar quick microtest was worked out by us, which deviates from his in certain essential points and which for that reason we shall describe here. The procedure used by Walch consists in putting a drop of a solution of the antibiotic on an inoculated serum agar plate, covering the spot with a cover glass, and subsequently incubating the plate. This procedure, however, permits only limited conclusions as to the sensitivity of the germs, because the antibiotic spreads by diffusion in the agar, so that a concentration gradient develops the details of which are unknown.

The procedure to be described here is based on the same microtechnique, but the antibiotic content in the nutrient medium is known and remains unchanged throughout the incubation period (subject to unavoidable small losses). Moreover, six different dilutions of the antibiotic are tested in one Petri dish. It will be shown that for the needs of the clinic the same accuracy is achieved as, say, in the test-tube test. A field without antibiotic serves as a growth control and permits a tentative bacteriological diagnosis (see below). For most germs a reading can be made after an incubation period of three to four hours; only rarely is more time required. If pleuropneumonia-like microorganisms and air forms, most of which are promoted by penicillin, are to be investigated, observations must be continued up to several days (Poetschke, 1951). For this purpose the serum content (see below) must amount to 20%.

The principle, as in Walch's method, is based on the microscopically detectable colony formation of the germs to be investigated as a criterion of their resistance, and the discontinued multiplication as a criterion of their

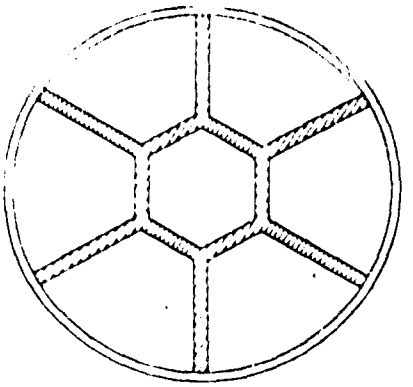

Figure 1. Diagram of a Petri dish with paraffin partitions for the quick microtest.

sensitivity. The reading is also done directly on the culture, covered with a cover glass and sealed with paraffin, with a phase constrast microscope. In principle the reading is possible with an ordinary bright-field microscope with adequate screening, but this is harder and does not give as clear, definite, well-contrasted images.

Technique

Sterile Petri dishes of about 90 mm in diameter, which should be thin-walled, unscratched, and as smooth as possible, are divided on the inside of the bottom with paraffin dams into seven separate fields (Figure 1). For this purpose a radial six-armed cross is drawn on the back and the seventh, central field drawn in on this division with a grease pencil. The melted paraffin is best applied to the inside with a soft brush along the grease-pencil marking. A pipette with a rubber cap may also be used, repeatedly warmed in the flame. The dams must be 1 to 2 mm high and must be run completely to the vertical edge of the dish and to each other.

To prepare the test nutrient medium, nutrient agar (on a meat broth or yeast extract base, or better still from digestive juices) is liquefied and then kept warm in a water bath of 48 to 50°, which can be improvised. A dilution series of the antibiotic in question is put into sterile test tubes, each 0.5 cm^3 containing five times the amount of the required final concentration; e.g. if a final concentration of 1 unit per cm^3 of nutrient medium is desired, 5 units dissolved in 0.5 cm^3 is put into the glass. What dilution series are chosen and whether the experimenter contents himself with six different concentrations is for him to decide. We now prefer the series shown in Tables 1 and 2. As a solvent and for preparation of the dilution series we take nutrient broth or grape-sugar (glucose) solution. To each 0.5 cm^3 of the dilution series 0.5 cm^3 of sterile human or equine serum or ascites is added. It is recommended that the serum be heated for one hour to 56 to 60° in order to destroy the antibacterial substances and to destroy as far as possible the bacterial contaminations which are always present. Tubes thus treated are put into the water bath

and 4 cm^3 of the liquefied agar heated to 48-50° C pipetted into each tube. This gives a 10% serum agar with a known concentration of the antibiotic. After repeated turning and rolling of the tube in the hand and brief heating in the water bath the nutrient medium is ready to pour. It is not practical to prepare more than 5 cm^3 of nutrient medium per tube. If more is needed it is better to start several series simultaneously. A tube with 5 cm^3 of nutrient medium suffices to pour six to eight fields; i.e. one will prepare six to eight plates and should then quickly pour all the fields in succession that are to contain the same concentration. The concentrations or at least the numbers of the fields should be written on the edge of the Petri dishes with a grease pencil. In order to avoid errors the dishes should be turned in the same direction before they are poured. The central field serves as a growth control and contains the same nutrient medium without antibiotic. The plates are ready for use immediately after the material sets. In the case of penicillin and streptomycin they can be kept two or three days in the refrigerator without significant change in the results, provided the antibiotic solutions were freshly prepared beforehand.

Inoculation of the fields with the germs to be tested or with materials from patients is done indirectly. Cover glasses (18 x 18 mm) previously well cleaned and degreased and sterilized dry, say in a Petri dish, are inoculated with the test material by placing a drop on one of them with a platinum wire loop, carefully laying a second cover glass upon it, and then taking the two cover glasses apart. They are laid on the nutrient medium fields with the inoculated side down. In this way very uniformly inoculated surfaces are obtained; this makes the results come out more uniform and also makes the reading easier. Very viscous material from the patients is diluted somewhat with nutrient broth. If the material proves to low in germs, it is incubated for three or four hours with a little broth and the test started after that time.

Uniform periods of incubation and uniform nutrient media give a uniform sensitivity of the test. In this test the sensitivity is of course also dependent on the germ density, though not to an interfering extent if the instructions are adhered to. In rush cases suspensions of solid cultures can be used, even in cases of mixed flora.

After all fields have been inoculated the nutrient medium which extends beyond the cover glass and the edge of the cover glass itself are covered with melted paraffin by means of a brush. The nutrient is protected against contamination and evaporation and consequently also against any gross change in concentration of the antibiotic.

Culture

The cultures are then incubated at 37° for about three hours. In general it is then possible to take a reading. Many germs show adequate colony formation after only two hours, many not until after four hours or more. Of the germs which are most frequently encountered it is chiefly Pseudomonas pyocyanea that requires four hours or more. Antibacterial therapy and long shipping times increase the required incubation period.

Besides the material to be tested, at least one microtest a day per antibiotic should be run with a well-known test germ as a control /see Note 1/. The incubation under the cover glass is anaerobic or at least takes place with greatly lowered oxygen level. To judge by our experience of some years, all pathogenic germs /see Note 2/ and most saprophytes grow under these conditions into colonies readily appraisable microscopically. For many genera and species the environment described here is more tolerable than cultivation on an open nutrient medium surface. The experienced bacteriologist knows that many a culture will succeed only in a liquid medium. Our microculture method is culture in a capillary liquid layer (usually less than 1 μ deep) between cover glass and agar surface. With good technique the germs grow at first in a single layer. Contaminations by air-borne germs have never yet shown a disturbing effect.

/Note 1/ Bacillus cereus is unsuited for this method because of its heavy colony formation. We use Staphylococcus aureus instead.

/Note 2/ Only the genus Mycobacterium (tbc) has not yet been tested.

Optics

Reading is done by setting the agar plates on the stage of the microscope and the desired field over the condenser. A mechanical stage to which the plate is fastened with plasticine greatly facilitates the work. The normal enlargement of the objective should be about 40 to a maximum of 60 times. The ocular should enlarge 10 to 12 times. We found the intermediate 1.6 x enlargement on the Zeiss-Winkel (Göttingen) standard microscope useful; it was eliminated in searching and used in examination. Mastery of the phase-contrast microscope is a prerequisite to rapid work. The phase condenser used must have a great enough width of section to penetrate the agar layer, but this only becomes critical in oil immersion.

Reading

Comparison with the test-tube test showed that colony formation is a usable criterion (see below). We classified the amount of multiplication into the following degrees (Figure 2):

Uninhibited growth (as in the growth control)	+ + +
Definite colony formation, but less than in the control	+ +
Slight multiplication without colony formation	+
Slight multiplication of individual germs, majority completely inhibited...	±
Complete inhibition	•

Inhibition of multiplication can be accompanied by an increase in volume (growth of the individual) and by a change in form. Thus, as is well known, under the influence of penicillin cocci may swell, bacilli lengthen and become distorted. These observations may be noted by circles of varying size combined with the other symbols, e.g. ⊖⊖ = moderate colony formation with great deformation, ϕ = no multiplication with slight deformation. Bommer (1950) has already made pictures of microcolonies of streptococci under the influence of penicillin and studied them cytologically.

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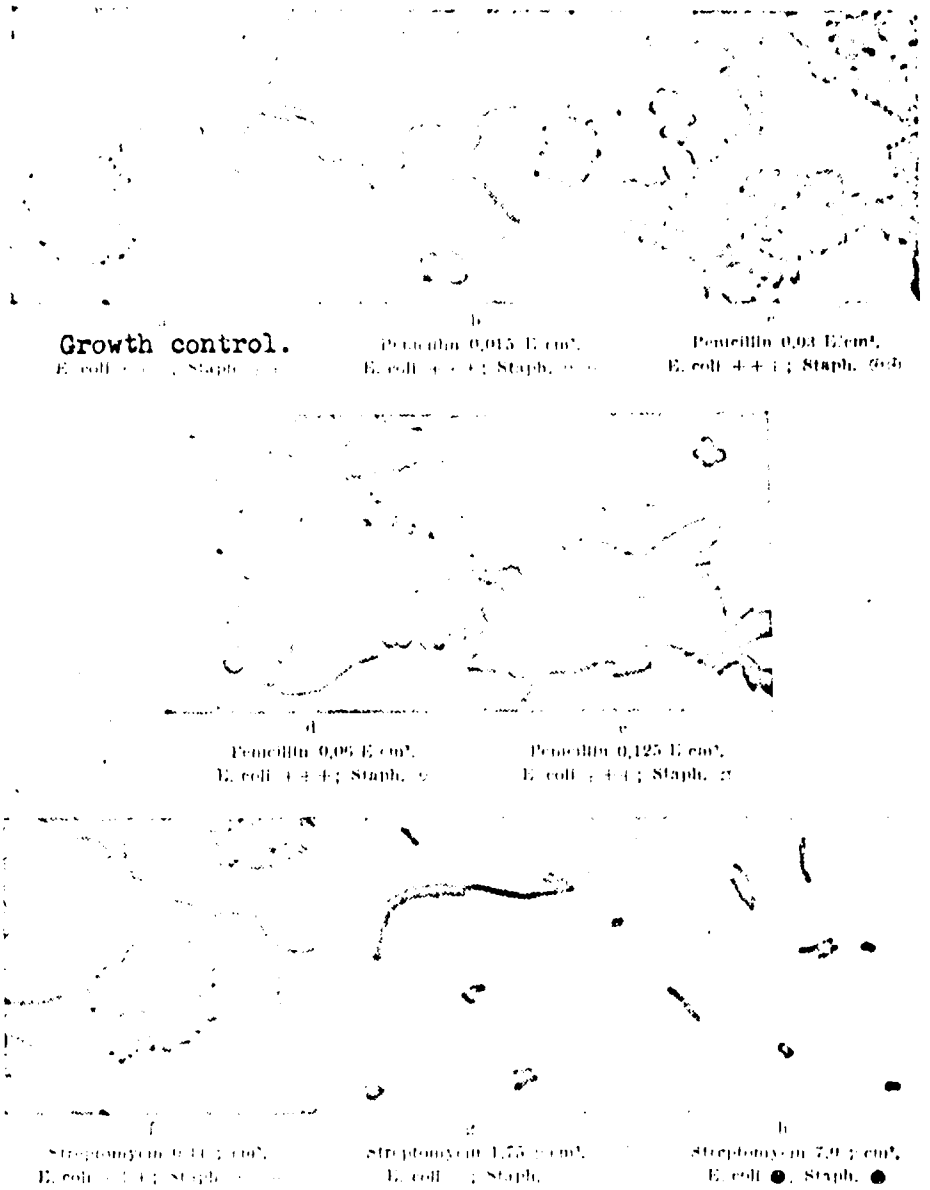

Figure 2 a-h. Escherichia coli and Staphylococcus aureus in mixed culture, 4 hours, 37° C. a) Growth control without antibiotic; b-e) with penicillin; f-h) with streptomycin. Phase contrast image, 1000:1. Objective Zeiss-Winkel objective Ph 40 x, photoocular 9 x. Explanation of symbols in text.

Evaluation

In order to be sure what microscopic image in the quick microtest (MST) corresponds to the macroscopic criteria of the test-tube test (RT) (+ = turbidity = resistance, - = clarity = sensitivity), parallel investigations were set up. Tables 1 and 2 show the results. It can be seen from Table 1 that in the case of streptomycin a very good correspondence between MST and RT usually prevails. The lack of morphologically provable divisions (● or ∅) corresponds to remaining clear in RT, though the latter often shows somewhat higher resistance values.

Table 1. (Explanation of symbols in the text.)

Bacterium and Test		Streptomycin in mg/cm³ 1,0	0,5	0,25	0,125	0,06	0,03	0,015	0,008	0,004	0,002	0,001	0,0005	Control	Strain
E. coli	MST							●	●	0	⊙	⊙	+++	+++	FDA
E. coli	RT	--	—	—	—	—	—	—	—	—	+	+	+	+	
Ps. pyocyan.	MST[1]	--		+		+		+		++		+++		+++	43282
Ps. pyocyan.	RT	-	+	+	+	++	+	+	+	+	+	+	+	+	
Staph. aur.	MST	o		●		⊙⊙		+++		+++		+++		+++	43283
Staph. aur.	RT	-	—	+	+	+	+	+	+	+	+	+	+	+	
Strept. faecal.	MST	o		+		+++		+++		+++		+++		+++	
Strept. faecal.	RT	--	—	—	+	+	+	+	+	+	+	+	+	+	44130
Strept. saliv.	MST	o		●		●		●		+++		+++		+++	
Strept. saliv.	RT	—	—	—	—	—	--	—	—	+	+	+	+	+	45276
E. coli	MST	o		o		●		++		++		+++		+++	
E. coli	RT	—	—	—	—	—	—	+	+	+	+	+	+	+	43585
E. coli	MST	o		o		o		●		+++		+++		+++	
E. coli	RT	—	—	—	--	—	—	--	+	+	+	+	+	+	4130

[1] Ps. pyocyanea usually grows slowly and can only be read after 4 to 8 hours.

Table 2. (Explanation of symbols in the text.)

Bacterium and Test		Penicillin in E/cm³ (E = units) 1,0	0,5	0,25	0,125	0,06	0,03	0,015	0,008	0,004	0,002	0,001	Control	Strain
Staph. albus	MST	●		●		⊙		⊙		⊙		+++	+++	K. H.
Staph. albus	RT	—	—	—	—	+	+	+	+	+	+	+	+	
Staph. albus	MST	++		++		++		+++		+++		+++	+++	44473
Staph. albus	RT	+	+	+	+	+	+	+	+	+	+	+	+	
Staph. albus	MST	o	o	o	⊙	⊙	⊙⊙						+++	47020
Staph. albus	RT	—	—	—	—	+	+	+	+	+	+	+	+	
Staph. aur.	MST	o	o	o	⊙	⊙⊙	⊙⊙						+++	47020
Staph. aur.	RT	—	—	—	—	+	+	+	+	+	+	+	+	
Staph. aur.	MST	o	o	●	●	●	⊙						+++	Bru.
Staph. aur.	RT	—	—	—	—	(+)	+	+	+	+	+	+	+	
Staph. aur.	MST	++	+++	+++	+++	+++	+++						+++	Ru.
Staph. aur.	RT	+	+	+	+	+	+	+	+	+	+	+	+	
Strept. faecal.	MST	●		●		⊙		⊙		+++		+++	+++	44473
Strept. faecal.	RT	—	--	—	—	—	+	+	+	+	+	+	+	
Staph. aur.	MST	o		●		⊙		⊙		⊙		+++	+++	A_1
Staph. aur.	RT	—	--	—	—	--	+	+	+	+	+	+	+	

The clinician will do well in the case of streptomycin to work for a level in the blood that amounts to twice or three times the inhibition value in the MST (as in fact he is accustomed to do with the RT).

In the case of penicillin matters are somewhat different. This antibiotic has a much greater effect on the morphology of the germ (deformation), and a less sharp demarcation of the inhibition. Concentrations that are far below the level of total inhibition suffice to lead to a considerable retardation of multiplication. This manifests itself in the fact that the MST, which works morphologically, shows several grades of division of the much inhibited

and usually deformed germs, while the RT no longer shows any turbidity. Here the MST may come considerably nearer the blood level clinically necessary than the RT.

The MST is also usable for aureomycin, terramycin, and chloromycetin, but as yet not much experimental work has been done.

In principle the MST is also usable for determination of the antibiotic content of body fluids, but further studies are necessary for that purpose. The same may be said for determination of resistance to other chemical therapeutics (sulfonamides).

To judge by experience up to now, the MST may be used equally well for all germs that can be grown in microculture.

If several MST's are started simultaneously from the same material, an excellent agreement of the tests among themselves is obtained.

The individual germs of mixed flora from normal or diseased organs can generally be read well in the same microculture at the same time. Within the first five or six hours we see no impairment of the effect of an antibiotic on a sensitive germ due to a different species of germ that destroys this antibiotic (e.g. Staphylococcus aureus in the simultaneous presence of Escherichia coli or Ps. pyocyanea under the influence of penicillin; see Figures 2a-e).

With regard to the theory of evaluation of sensitivity values let us emphasize here that only the following conclusion is sure: A germ resistant in vitro to a certain concentration will be so in vivo as well. On the other hand a germ sensitive in vitro to a certain concentration need not be so in vivo. Nor do we possess any criterion as to what increase in the level in the blood over the sensitivity limit in vitro is capable of so inhibiting a germ in its host that a therapeutic effect is sure to be achieved. Schuermann and Schirduan (1948) in their valuable investigation of the dependent relationship between penicillin dosage and rate of recovery showed that with an increase in the dose only the probability of a cure increases. Approach to the value of 100% cures does not occur until very high dosages. Briefly, then, no reliable conclusion as to the level in the blood that is therapeutically required can be drawn from the sensitivity in vitro. The measure of sensitivity, furthermore, is only a very crude indication for dosage. Statements about the lowest therapeutically effective level in the blood (say 0.03 units of penicillin per cubic centimeter) are untenable. The report of a single definite inhibition value for a species of germ, strictly speaking, is an abstraction. This very method of ours shows that bacterial populations have a frequently very wide spread of inhibition values of single individuals. It is hardly to be assumed that this is due to acquisition of resistance within a few hours. For clinical practice it is the maximum inhibition value for a germ species that is of greatest interest.

Bacteriological Diagnosis

The question whether the individual species of germs form such characteristic microcolonies as to permit a tentative diagnosis on this basis has caused

to delay publication of the microtest for more than a year. The answer to this question is neither affirmative nor negative. Some species show such characteristic forms that the classification has a high degree of reliability; e.g. pneumococci, most streptococci, H-form of proteus, capsule bacteria, pasteurella, Corynebacteria, actinomycetes, streptomycetes. In other species the form is less reliable and permits only rough groupings; e.g. "bacilli, probably E. coli." Now the conjectural diagnosis can be narrowed down by carefully lifting the cover glass of the growth control and staining by Gram's method. The provenience of the material also often facilitates classification.

The reliability of the diagnosis is dependent on the general and specific experience of the investigator and his morphological talents.

It is our intention to show characteristic microcolony patterns in a publication to appear in the near future.

We believe that in spite of the tentative nature of the bacteriological diagnosis in microculture the clinic and general practice are better served by learning something about the probable flora and their sensitivity to antibiotics four to six hours after the materials are collected than by practicing antibiotic therapy blind for 24 to 48 hours and perhaps spending time, material, and money without helping the patient.

Whether the test will make a place for itself in the clinical laboratory or whether it needs a trained bacteriologist time will tell.

Summary

A quick microtest on a morphological basis, developed independently of Walch, is described which in three to five hours shows the sensitivity of germs and mixtures of germs to antibiotics and permits a conjectural bacteriological diagnosis. For clinical purposes the accuracy is equal to that of other testing methods. The sensitivity of the germs to antibiotics can be given in figures.

Bibliography

Bommer, W., medical dissertation, Marburg, 1950.

Poetschke, G., Tagungsberichte der Deutschen Gesellschaft für Hygiene und Mikrobiologie (Minutes of the Meeting of the German Society for Hygiene and Microbiology), 1951.

Poetschke, G., Zentralblatt für Bakteriologie (Central Journal of Bacteriology), I, original articles (in preparation).

Poetschke, G. and W. Bommer, Zentralblatt für Bakteriologie, I, orig., Vol 156, 1950, page 128.

Schuermann, H. and M. Schirduan, Klinische Wochenschrift (Clinical Weekly), Vol 26, 1950, page 526.

Walch, E., Klinische Wochenschrift, 1951, page 540.